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EURL Lm

European Union Reference Laboratory for

*Listeria monocytogenes*

# EURL *Lm* TECHNICAL GUIDANCE DOCUMENT

## for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods

**Version 3 – 6 June 2014**

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CONTENTS

## Foreword

This document is the third version of the Technical Guidance Document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods of the European Union Reference Laboratory for *Listeria monocytogenes* (EURL *Lm)*. It replaces the 2nd version of November 2008.
This document is complementary to the EC/DG SANCO document, entitled "Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs".

This document has been approved by the Standing Committee of the Food Chain & Animal Health/Section "Biological safety of the food chain", at its meeting of 16 June 2014.

References are given in clause 5 and annexes in clause 7.

# 1 Introduction

## *1.1 Listeria monocytogenes*

The genus *Listeria* contains ten species including *monocytogenes*, which may be pathogenic for humans and animals. *Listeria monocytogenes* may cause a disease called listeriosis that may affect humans and animals.
*Listeria* is a small (0.5-2 μm x 0.5 μm), Gram-positive bacillus, isolated or arranged in small chains, motile at 20-25°C and non-spore-forming. It is aerobic and facultatively anaerobic, catalase-positive except for a few rare strains, oxidase negative and hydrolyses esculin. *Listeria* ferments many carbohydrates without producing gas. Strains of *L. monocytogenes* are always D-xylose negative and produce lecithinase. They are generally β-haemolytic and L-rhamnose positive. The species *monocytogenes* is divided into 13 serovars based on somatic and flagellar antigens. Since 2005, these serovars have been replaced by 5 genoserogroups determined by PCR: IIa (serovars 1/2a and 3a), IIb (serovars 1/2b and 3b), IIc (serovars 1/2c and 3c), IVb (serovars 4b, 4d and 4e) and L (other serovars). Of these, IVb followed by IIa and IIb are the genoserogroups most frequently implicated in human cases.
Table 1 describes some growth and inactivation characteristics of *L. monocytogenes*.

**Table 1: Some growth and inactivation characteristics of *L. monocytogenes* (variable depending on the strains and the food matrix)[1]**

| | Growth | | |
|---|---|---|---|
| | Min. (lower growth limit) | Optimum (fastest growth) | Max. (upper growth limit) |
| **Temperature (°C)** | -1.5 | 30.0-37.0 | 45.0 |
| **pH** | 4.2-4.3 | 7.0 | 9.4-9.5 |
| **$a_w$** | 0.93 (0.90 with glycerol) | 0.99 | > 0.99 |
| **Salt concentration (%)[2]** | < 0.5 | 0.7 | 12-16 |
| **Atmosphere** | Facultative anaerobe (it can grow in the presence or absence of oxygen, e.g. in a vacuum or modified atmosphere package) | | |
| | **Thermal inactivation** | | |
| **$D_{65°C}$** | 0.2 to 2 min | | |
| **z** | 7.5°C (4 to 11°C) | | |
| | **High pressure inactivation** | | |
| 400 MPa for 10 min at 20°C → 2 $\log_{10}$ reductions in phosphate buffer (pH 7) | | | |
| 400 MPa for 10 min at 20°C → 8 $\log_{10}$ reductions in citrate buffer (pH 5.6) | | | |
| 400 to 500 MPa for 5 to 10 min at 20°C → 3 to 5 $\log_{10}$ reductions in meat products. | | | |
| 350 MPa for 5 to 10 min at 20°C → 3 to 5 $\log_{10}$ reductions in acidic products (e.g. fruit juices, jams). | | | |

Sources: Anses datasheet on biological hazards "*Listeria monocytogenes*", 2011 and Guidance document on *Listeria monocytogenes* shelf-life studies for ready to eat foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

[1]: The growth and inactivation data from *L. monocytogenes* presented in this table are based on research carried out primarily in lab media under optimum conditions and may vary depending on the strain and food matrix

[2]: Based on percent sodium chloride, water phase

*L. monocytogenes* is a psychrotrophic bacterion which is able to grow at -1.5°C, and thus may grow well at refrigeration temperatures. The microorganism has the ability to persist in food-processing areas and equipment.

Listeriosis occurs in two forms: invasive or non-invasive. For the entire population, listeriosis can cause bacteremia, septicemia, meningitis, meningoencephalitis, rhombencephalitis, brain abscess, local infections. In addition, for pregnant women, listeriosis can provoke flu-like symptoms (fever, chills, back pain), spontaneous abortion, death in utero, prematurity and neonatal infection. Susceptible population groups are people most likely to develop a severe form of listeriosis. These groups are composed of pregnant women, people aged over 65 years or those with cancer or blood disorders, dialysis patients, other immunocompromised diseases (e.g. HIV, …). Non-invasive forms are rare: they are essentially febrile gastroenteritis, for which some outbreaks have been recorded.

Although rare, listeriosis is a foodborne infection with extremely high lethality (25 to 30%) and hospitalisation rates (>92%).

## 1.2 Ready-to-eat foods

The definition of ready-to-eat food is given in the glossary (clause 6) and is taken from Regulation (EC) 2073/2005 of 15 November 2005 (modified) on microbiological criteria for foodstuffs and DG SANCO "Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs".

## 1.3 Legislative background

The first version of this technical guidance document (2008) was prepared at the request of Directorate General Health & Consumers (DG SANCO) of European Commission (EC) in response to the needs expressed by EU Member States. EC/DG SANCO acknowledged that a document was required, providing both detailed and practical information on how to conduct shelf-life studies on *Listeria monocytogenes* (*L. monocytogenes*) in ready-to-eat foods to ensure conformance to the microbiological criteria set out in Article 3.2 of Regulation (EC) No. 2073/2005.

Annex I of Regulation (EC) No. 2073/2005 sets out the microbiological criteria for foodstuffs, including the criteria for *L. monocytogenes* in RTE foods (criteria 1.1 to 1.3). Annex II of this regulation specifies that food business operators (FBOs) shall conduct, as necessary, studies to evaluate the growth of *L. monocytogenes* that may be present in the product during the shelf-life under reasonably foreseeable storage conditions. Annex II does not describe the technical procedure to conduct such studies.

## 1.4 EU guidance document dedicated to food business operators

The EC/DG SANCO document, entitled "Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs", is directed at FBOs who produce ready-to-eat foods. In this document, a decision tree shows a schematic approach for the steps of shelf-life studies, giving the FBO an indication of when additional specific studies are needed in order to investigate the growth of *L. monocytogenes* in the product.

# 2 Scope

This EURL *Lm* technical guidance document is basically intended for laboratories conducting challenge tests and durability studies on *L. monocytogenes* in RTE foods, on behalf of the FBOs. These laboratories should have the required expertise for such studies and demonstrate good laboratory practices.

This document describes laboratory studies, challenge tests and durability studies related to the growth of *L. monocytogenes* in RTE foods.

It is mainly dedicated to packaged products[1]. For unpackaged products, other additional factors, such as hygrometry, have to be considered for the storage of the product under reasonably foreseeable storage conditions; it is thus necessary to adapt the review of data and the experimental protocol to this type of products.

The shelf-life is determined for the product as marketed by the producer. Once the product is opened and stored by a retailer, restaurant owner,…, a new shelf-life has to be assessed.

This document details the relevant information required before implementing a challenge test and recommendations on how to implement and perform the challenge tests required: challenge test assessing growth potential or challenge test assessing maximum growth rate.

This document also provides recommendations on how to implement and perform durability studies.

The choice of the test to be implemented should be done by the FBO, if necessary with the collaboration of the laboratory which will conduct it. The choice should be based on the information to be obtained, as illustrated in Figure 1.

[1] Challenge tests for packaged products should be conducted using the product in its final packaged format including gas atmosphere if present. For products which are intended to be displayed in bulk (i.e. large blocks of cheese, pieces of ham or tubs of deli-salads), the tests should be conducted using the typical packaging which is expected to be supplied to consumers (e.g ham may be overwrapped with packaging film, salads may be filled into plastic pots). The aim of a challenge test is to simulate as closely as possible the likely storage conditions of the product. The challenge test report should record what packaging and storage conditions were used as the results are not applicable to different storage conditions.

Figure 1 describes microbiological procedures for determining the growth of *L. monocytogenes* using challenge tests and durability studies.

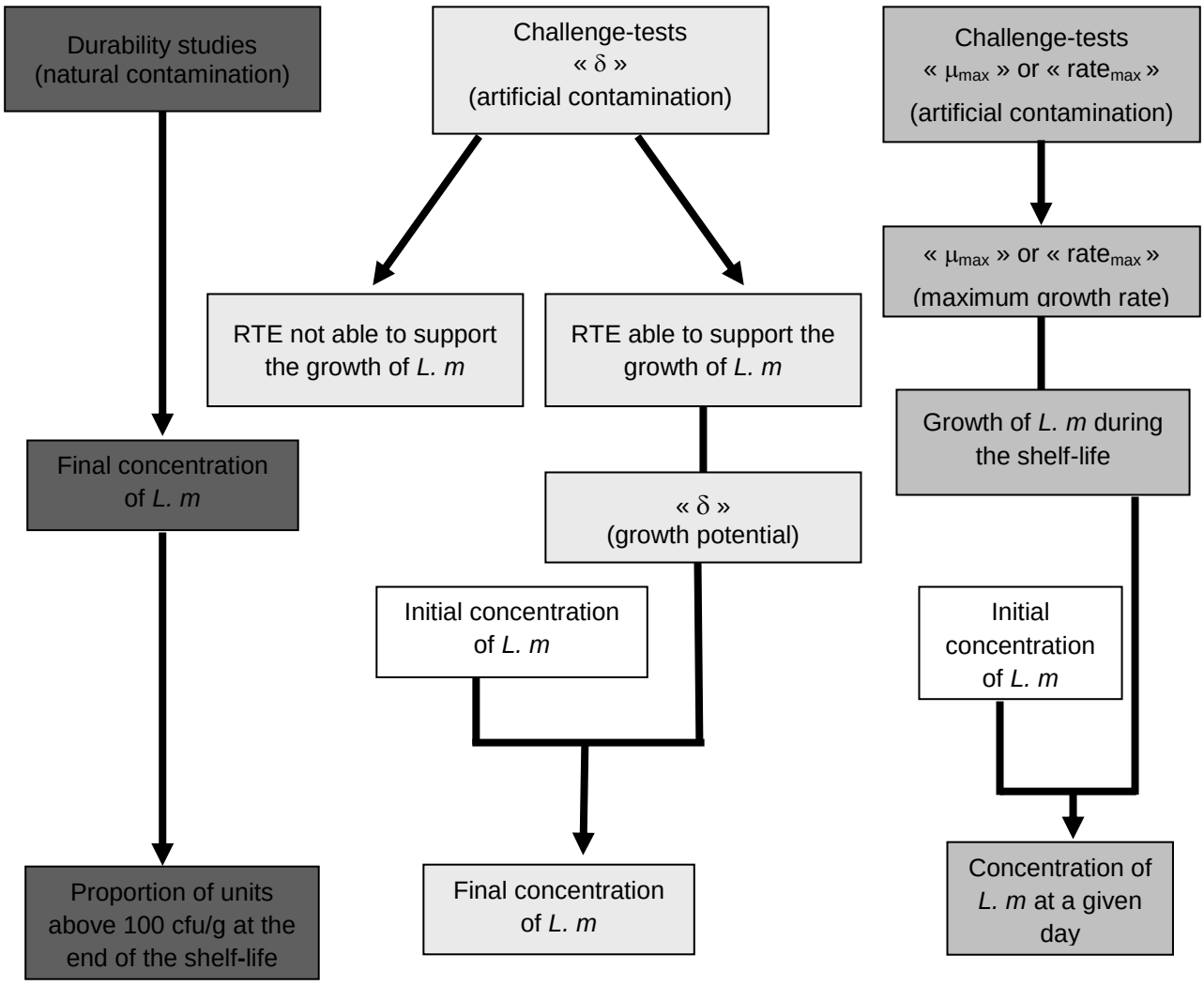



**Figure 1. Data obtained from shelf-life studies**

Challenge tests aim to provide information on the behaviour of *L. monocytogenes* which have been artificially inoculated into a food, under given storage conditions. They may take into account the variability of the batches, of the food samples and of strains. The level of contamination, the heterogeneity of the contamination and the physiological state of the bacteria are difficult to mimic in a challenge test study; the contamination method cannot always enable to fully imitate the natural contamination.

Durability studies allow an assessment of the shelf-life of the food regarding *L. monocytogenes* in a naturally contaminated food during its storage according to reasonably foreseeable conditions. Durability studies may be considered more realistic than a challenge test, as the contamination is naturally occurring. But the implementation of durability studies is limited in case of low prevalence and low level of contamination.

# 3 Challenge test

If the challenge test is related to a range of products, only the product which is expected to give the worse-case scenario for *L. monocytogenes* growth is tested. This product is selected by the FBO, possibly with the help of the laboratory.

A flow diagram describing schematically the different steps, from review of data to laboratory procedure, is given in clause 7.1.

## 3.1 Review of data

Primarily, the FBO is responsible for setting the shelf-life under defined conditions, which should take into account reasonably foreseeable conditions during transportation[2], storage at retail and at consumer levels.

The FBO should be able to provide the laboratory with the following relevant product information in order to define the experimental challenge testing procedure when a challenge test is needed:

- Description of the product.
- Composition of the RTE food.
- Product characteristics; it is important to note if the values of certain characteristics change during the shelf-life.
- Packaging condition of the end-product.
- The main steps of the production process.
- Characterisation of the cold chain.

[2] From manufacturer to retail, including storage in the warehouses.

## 3.2 Protocols for challenge test

A challenge test should be performed using the conditions which would be the most favourable for growth of *L. monocytogenes* within the possible range of each parameter.

Two types of challenge test can be distinguished: a challenge test assessing growth potential (3.2.1) and a challenge test assessing maximum growth rate (3.2.2).

### 3.2.1 Challenge test assessing growth potential

#### *3.2.1.1 General aspects*

A microbiological challenge test assessing a growth potential ($\delta$) is a laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under foreseeable conditions of transportation, storage at retail and at consumer levels. A microbiological challenge test has to reflect the foreseeable conditions that might be expected to occur throughout the cold chain, including storage conditions between production and consumption. The test period starts the day of contamination and finishes at the end of the shelf-life[3].

The growth potential ($\delta$) is the difference between the $\log_{10}$ cfu/g at the end of the test and the $\log_{10}$ cfu/g at the beginning of the test.

$\delta$ depends on many factors, the most important being:

- the inoculated strain(s),
- the inoculation level,
- the physiological state of the inoculated cell(s),
- intrinsic properties of the food (e.g. pH, NaCl content, $a_w$, associated microflora, antimicrobial constituents),
- extrinsic properties (e.g. time-temperature profile, gas atmosphere, moisture).

In the frame of the implementation of the Regulation (EC) No. 2073/2005, $\delta$ can be used:

- to classify a food:
  - when $\delta > 0.5$ $\log_{10}$ cfu/g, the food is classified into "Ready-to-eat foods able to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes" (category 1.2),
  - when $\delta \leq 0.5$ $\log_{10}$ cfu/g, the food is classified into "Ready-to-eat foods unable to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes" (category 1.3),
- to quantify the behaviour of *L. monocytogenes* in a food of category 1.2 according to defined reasonably foreseeable conditions between production and consumption.

[3] The contamination should be performed within 2 days after the production day.

The main advantage of a challenge test assessing growth potential is that the lag phase related to the temperature is taken into account. In this test, stresses or adaptation could be applied to the cells and impact the lag phase.

The drawback of this test is the lack of flexibility in the interpretation: the results are only valid for the product tested under specific conditions, so that new experiments have to be performed each time there is a change (e.g. use of different time-temperature profiles, …).

#### *3.2.1.2 Protocol of a challenge test to assess growth potential*

##### a. Number and choice of batches

- Number of batches

The number of batches to be tested can be determined using a growth/no growth boundary module of a predictive microbiology software and/or using a calculator to determine the inter-batch variability (see below).

Batches have to be the batches that are the most favourable to *Lm* growth.

- **Use of a growth/no growth boundary module of a predictive microbiology software**

Predictive microbiological models have to be used with caution and only used by staff with expertise and an understanding of their limitations and conditions of use.

Using a growth/no growth boundary module (see Figure 2), it is possible to get the growth probability of *Listeria monocytogenes* according to pH, $a_w$ and temperature. In the case of no growth or low growth probability (≤ 10%), it is possible to limit the study to one batch.

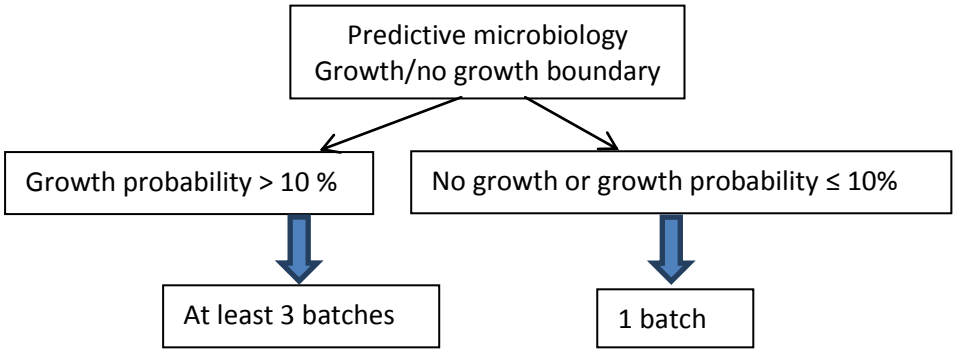



**Figure 2: Decision tree for the number of batches in the implementation of a challenge test by using predictive microbiology**

### ➢ Use of a calculator to determine the inter-batch variability

The calculator "inter-batch variability" developed by EURL *Lm* and available at http://www.ansespro.fr/eurl-listeria/index.htm (see Figure 3) enables one to test the inter-batch variability of the physico-chemical characteristics of the product related to *L. monocytogenes*. It is based on:

- physico-chemical characteristics (pH, $a_w$) of at least 3 batches;
- storage temperatures;
- cardinal values of the strains: $T_{min}$ (minimum growth temperature), $T_{opt}$ (optimum growth temperature), $pH_{min}$ (minimum growth pH), $pH_{opt}$ (optimum growth pH), $a_{w_{min}}$ (minimum growth $a_w$), $a_{w_{opt}}$ (optimum growth $a_w$).

Note that this calculator takes into account only two physico-chemical parameters (pH and $a_w$). However, if other characteristics may have an impact on the growth of *L. monocytogenes*, they have to be also taken into consideration.

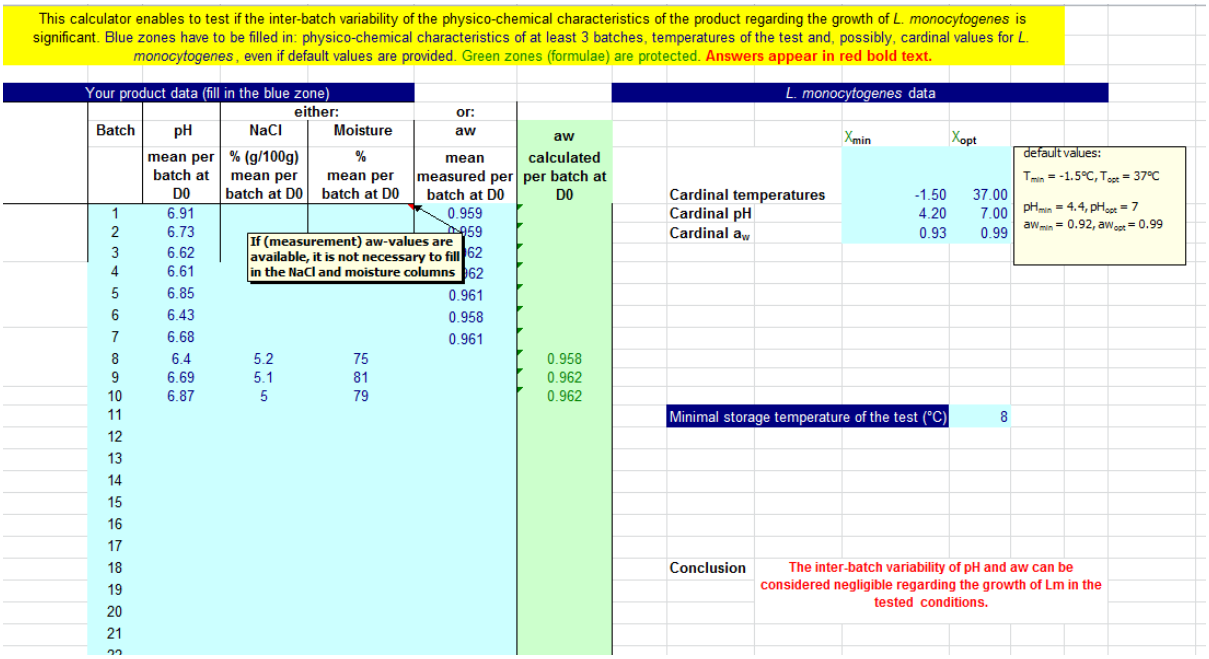



**Figure 3: Example of the use of the calculator to determine the inter-batch variability of the physico-chemical characteristics (Excel file)**

**Example**

Data relating to the strains

Cardinal values are unknown, so default values for *L. monocytogenes* are provided in the calculator.

Data relating to the food

Note that, if $a_w$ values are not available, they can be replaced by estimated values, calculated by the calculator on the basis of the water phase salt content WPS (in g/100ml):

$$WPS = \frac{salt\ content\ (in\ g\ per\ 100g)}{moisture\ content\ (in\ ml\ per\ 100g)}\ x\ 100$$

with the following equation:

$$a_w = 1 - 0.0052471\ x\ WPS - 0.00012206\ x\ WPS^2$$

This formula is based on the salt content but other components can modify the values of $a_w$.

Storage temperature relating to the test:

For example T = 8°C at FBO and T= 12°C at retail and consumer

Result given by the calculator (see Figure 4)

- If the inter-batch variability of pH and $a_w$ can be considered negligible regarding the growth of *L. monocytogenes* in the tested conditions, then it is possible to limit the study to 1 batch. It is sufficient to use 1 single batch, except if the variability of the characteristics other than pH and $a_w$ may have an impact on the growth of *L. monocytogenes*.
- If the inter-batch variability of pH and $a_w$ has a significant impact regarding the growth of *L. monocytogenes* in the tested conditions, then at least 3 batches must be tested.

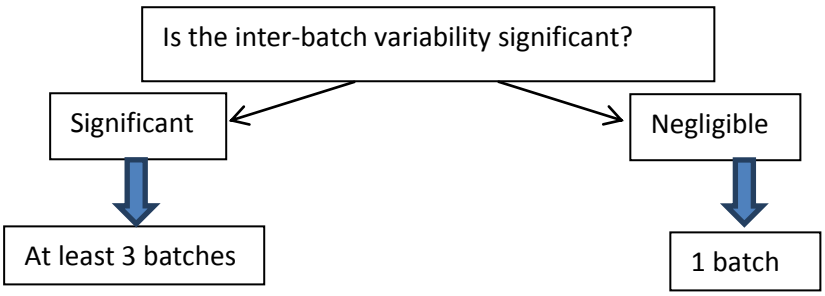



**Figure 4: Decision tree of the number of batches in the implementation of a challenge test by using the calculator**

- Choice of batches

When at least 3 batches have to be tested, the batches with the physico-chemical parameters (pH, $a_w$) most favourable to growth, produced in normal conditions of the manufacturing process and not at the same time, will be analysed.

## b. Choice of strains

To account for variation in growth and survival among strains of *L. monocytogenes*, a challenge test should generally be conducted with a pool of strains. Using an inoculum of multiple strains of a given pathogen is preferred, as it will help to encompass the variability among bacteria.

### o Number and choice of strains

Challenge tests assessing growth potential are performed with a mixture of at least 2 strains to account for variations in growth among the strains. One of them has to be a strain with known growth characteristics. The other strain(s) is (are) freely chosen (for example: from foods, environment, outbreak, collections); knowledge of the growth characteristics is not mandatory for this (these) strain(s).

Growth of *L. monocytogenes* strains varies depending on the food and storage conditions studied. To help the laboratory to choose *L. monocytogenes* strains, EURL *Lm* has constituted a set of strains of *L. monocytogenes* isolated from different origins (meat, fish, milk products and other origins). These strains have been characterised for their growth abilities ($\mu_{max}$ have been determined in harsh pH, $a_w$ and temperature conditions (see clause 7.2)). Examples of how to select an EURL *Lm* strain are also given in clause 7.2. The EURL *Lm* set of strains is made available to the NRLs.

**Note**: If it is necessary to conduct a challenge test at a pilot scale, non-pathogenic surrogate organisms have to be used. The surrogate being used should demonstrate growth characteristics equal to that of *L. monocytogenes*. For example, *Listeria innocua* can be used as a surrogate for *L. monocytogenes*.

### o Strain storage and characteristics

*L. monocytogenes* should be stored in the laboratory by a method which minimises or eliminates mutations which may affect their growth or survival characteristics.

Growth, biochemical and serogenotypic characteristics should be checked occasionally.

### c. Preparation of the inoculum

Each strain is subcultured twice:

- Firstly, in a medium (e.g. Tryptone Soy Broth (TSB) or Brain Heart Infusion (BHI)) and at a temperature (30 or 37°C) favourable to optimal growth of *Listeria monocytogenes*, for a sufficient time for the organism to reach the early stationary phase (for strains of EURL *Lm* set: during 15-18h) (see Figure 9 in clause 7.3.1). This first subculture is mainly aimed at getting the cells in the same physiological state.
- Secondly, at a temperature close to the storage temperature of the product (for example 7°C, 10°C), in order to adapt the strain to the storage condition of the product. This culture is incubated for a sufficient time necessary to reach the early stationary phase, to shorten the lag phase once inoculated in the product (for example for strains of EURL *Lm* set: during 7 days at 7°C or 3 days at 10°C).

Then, each second subculture is combined in equal quantity (see Figure 10 in clause 7.3.2). From the mixed culture, successive dilutions are prepared in physiological water at the temperature of the second subculture (for example 7°C, 10°C) to obtain an inoculum at the expected concentration (an example of calculation is explained in clause 7.3.4). The inoculum has to be used immediately.

The targeted inoculum level is checked by enumeration on a selective agar.

Stresses and adaptation of process are not taken into account in this document because it depends on the process and the food concerned. Extra stresses may be added if required.

### d. Preparation and inoculation of the test units

Table 2 shows the minimum number of test units to be prepared per batch.

The whole experiment requires destructive sampling for microbiological procedures.

**Table 2: Minimum number of test units to be prepared per batch**

| | "day 0"[a)] | "day end"[b)] |
|---|---|---|
| Enumeration of *L. monocytogenes* in inoculated test units | 3 | 3 |
| Detection of *L. monocytogenes* in non-inoculated test units | 3 | 3 |
| Measurement of physico-chemical characteristics in non-inoculated test units | 1 | 1 |
| Measurement of gas atmosphere (for product under gas atmosphere) in non-inoculated test units | | |
| Enumeration of the associated microflora in non-inoculated test units | | |

a) "day 0": the day of inoculation

b) "day end": the end of the shelf-life

In Table 2, only "day 0" and "day end" are considered, but it is highly recommended to add intermediate analysis points to consider[4] the variability of the matrix (an example of the number of units required is given in clause 7.4).

For measurement of physico-chemical characteristics, gas atmosphere and determination of the concentration of the associated microflora, the same test units may be used.

### o Preparation and inoculation of test units with *L. monocytogenes*

*Inoculation procedure*

The inoculation step is a critical step in the performance of a challenge test.

The inoculation has to be as effective as possible maintaining the intrinsic properties of the food. In order to minimise changes in the physico-chemical properties, the inoculum volume should not exceed 1% of the mass (or volume) of the test unit, otherwise it can seriously affect the intrinsic properties of the product and thus the growth characteristics of the inoculum (see clause 7.3.4). The inoculation mimics realistic conditions but the inoculum is homogeneously distributed in the food, even if in reality this may not be the case.

It is recommended to inoculate as follows:

- ✓ Either the food is removed from its packaging, inoculated and then repacked under similar gas conditions as an unopened pack (consumer pack). The RTE food can be inoculated:
  - in depth: for food considered to be homogeneous (e.g. ground food) or food prepared by mixing several materials (e.g. mixed salad),
  - at surface: to mimic contamination of a specific part during process (e.g. products contaminated during slicing).

    For products having multiple components or layers, one or few relevant components regarding *L. monocytogenes* contamination and/or the interfaces between components should be inoculated (for example: sandwich).
- ✓ Either the food is maintained in its packaging and so the contamination is possible only at surface, by inoculating through a septum which is immediately recovered by a second septum to maintain exact gas conditions.

Some examples of different contamination techniques are detailed in clause 7.5 It is desirable to test the contamination technique with a diluted dye before the inoculation with *Listeria monocytogenes* to visualise the dispersion of the volume inoculated. Other techniques can be used if it can be demonstrated that the moisture content is not changed and will not affect intrinsic properties of the food (for example: dipping).

[4] It is also suggested to perform analyses to take into account possible peaks of *L. monocytogenes* (a fast increase of *L. monocytogenes* in the product followed shortly by a fast decrease of *L. monocytogenes)* from production until consumption (knowing that these possible peaks are close to the beginning of the shelf-life).

Care should be taken to ensure that headspace volume and gas composition of the challenge test samples mimic the commercial food product as closely as possible.

*Contamination level*

The contamination level is targeted at around 100 cfu/g. This reduces the effect of measurement uncertainty.

### o Preparation of non-inoculated test units

*Detection of* L. monocytogenes *at day 0*

No physiological water is added to test units used to detect *L. monocytogenes*. If *L. monocytogenes* is present in these test units, the result of the challenge test is not valid.

*Physico-chemical characteristics and associated microflora*

In the test units used to determine the physico-chemical characteristics and the concentration of the associated microflora, it is necessary to inject a volume of sterile physiological water identical to the volume of the *L. monocytogenes* inoculum.

The determination of the physico-chemical characteristics and associated microflora is necessary to compare the products submitted to challenge testing to the products routinely produced by the factory (see data required in clause 3.1). Moreover, pH measurement may explain the behaviour of *L. monocytogenes*. Determination of the concentration of the associated microflora can provide additional information about possible interactions between *L. monocytogenes* and associated microflora. Such interactions may influence the growth of *L. monocytogenes*.

### o A challenge strain control

To demonstrate whether storage conditions are restricting the growth of the *L. monocytogenes* strains or whether it is the food matrix itself, the growth of these *L. monocytogenes* strains is checked individually by an inoculation of each strain into a non-selective broth incubated under the same storage conditions as the samples in the challenge test. Growth of the strains is checked by turbidity measurement or by enumeration.

## e. Storage conditions

### o Introduction

The storage (incubation) conditions applied during challenge testing have to comply with the conditions at which the product is most likely to be subjected in normal use, until its final consumption. This should include the typical temperature range along the cold chain: from the manufacture to retail, storage at retail and storage at consumer.

Temperature during shelf-life is a critical part of this challenge test (see clause 7.6). It is the responsibility of the FBO to ensure that the storage conditions used are realistic, taking into account that storage temperatures labelled on the packaging could not always be maintained throughout the cold chain (from production to consumption). If an inappropriate storage temperature (lower temperature than the usually encountered) is used, there may be an underestimation of *L. monocytogenes* growth and an overestimation of the safe shelf-life length. It is the reason why challenge tests have to consider the use of abuse temperature(s).

### o Storage temperature and duration

The temperature(s) used to determine shelf-life of the product has (have) to be properly justified and documented by the FBO.

- When the FBO has its own data on the two first stages of the cold chain (from manufacturing to retail, storage at retail), the use of this information is preferred (in particular for exported products). In this case, use the $75^{th}$ percentile of own FBO data's observation.
- If no FBO data is available, Table 3 has to be followed.

**Table 3: Flow diagram of storage (incubation) conditions**

| Stage of cold chain | Storage (incubation) temperature | | | Storage (incubation) duration | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | Shelf life ≤ 21 days | Shelf life > 21 days |
| From the manufacture until the arrival to the display cabinet | Temperature justified by detailed information* | Or if not known | 8°C | Duration justified by detailed information | Or if not known | One third of the total shelf life of the product | 7 days |
| Retail: Display cabinet | Temperature justified by detailed information* | Or if not known | 12°C | Duration justified by detailed information | Or if not known | One third of the total shelf life of the product | ½ (shelf life – 7 days) |
| Consumer storage | Temperature justified by detailed information* | Or if not known | 12°C | Duration justified by detailed information | Or if not known | One third of the total shelf life of the product | ½ (shelf life – 7 days) |

* Temperature justified by detailed information: the 75$^{th}$ percentile of the observations for the country where the stage of the cold chain is located.

### f. Measurement of physico-chemical characteristics

The physico-chemical characteristics (at least pH; [NaCl content, moisture] or $a_w$) are measured according to standard methods. Other factors, such as organic acids, nitrite, fat, can influence the behaviour of pathogens and could be measured at least at the beginning and at the end of the challenge test.

### g. Gas atmosphere

For test units conditioned under modified atmosphere or vacuum packed, it is desirable to pay attention to:

- the gaseous composition at "day 0" and at "day end" (as for the physico-chemical characteristics) or
- the performance of the packaging machine in case of repacked products in the laboratory and the gas permeability of the packaging.

## h. Microbiological analyses

The laboratory conducting challenge tests should preferentially be accredited to EN ISO 17025 for the detection and enumeration of *L. monocytogenes* in food. For other microbiological parameters, such as other microorganisms useful to better interpret the results of the challenge test, assessment of validity of analyses through regular participation to proficiency testing trials for other bacteria than *L. monocytogenes* is sufficient.

### o *Lm* detection methods

According to Annex I of Regulation No. 2073/2005, the reference method for detection of *L. monocytogenes* is the Standard method EN ISO 11290-1, amended. According to Article 5 of the same regulation, the use of alternative analytical methods is acceptable when the methods are validated against the reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO Standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the Competent Authority.

### o Enumeration methods

According to Annex I of Regulation No. 2073/2005, the reference method for enumeration of *L. monocytogenes* is the Standard method EN ISO 11290-2, amended. According to Article 5 of the same regulation, the use of alternative analytical methods is acceptable when the methods are validated against the reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO Standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the Competent Authority.

The initial suspensions are prepared, when possible, by using the entire contaminated sample (see clause 7.7).

Since the targeted contamination level is 100 cfu/g, it is recommended to lower the limit of enumeration at 10 cfu/g, by according to EN ISO 11290-2:

- using 1 ml of the initial suspension spread onto 3 plates of ∅ 90 mm, or spread onto 1 large plate of ∅140 mm,
- or, for validated alternative methods, pour-plated into 1 plate of ∅ 90 mm.

**Note**: For a liquid matrix the limit of enumeration of the Standard method is at 10 cfu/g.

The associated microflora that may be taken into account include a mesophilic aerobic count or a specific microflora of the food (e.g. lactic acid bacteria, *Pseudomonas*, yeasts, moulds). Methods used to enumerate these associated microflora should follow relevant CEN, ISO or national standards for the organism and food type concerned.

### i. Calculation of growth potential

The growth potential ($log_{10}$ cfu/g) is estimated in the frame of this document as the difference between the median of results at the end of the challenge test and the median of results at the beginning of the challenge test.

Once each batch unit has been inoculated and enumerated at "day 0", it is recommended to calculate immediately the standard deviation between the $log_{10}$-results at "day 0". If this standard deviation (due to measurement uncertainty and contamination heterogeneity) is higher than the limit of 0.5 $log_{10}$ cfu/g, then the challenge test is not acceptable.

For each batch, the growth potential δ is calculated and the highest δ value obtained is retained amongst all tested batches.

A first example of results is shown in Table 4.

**Table 4. First example of results obtained from a growth potential test.**

CONVERSION in $log_{10}$

| Batches | Day | Concentration (cfu/g) | Concentration ($log_{10}$ cfu/g) **In bold: median** | Growth potential "δ" per batch ($log_{10}$ cfu/g) | Highest"δ" among the 3 batches ($log_{10}$ cfu/g) |
|---|---|---|---|---|---|
| 1 | "day 0" | 140 | **2.15** | 2.11-2.15=-0.04 | **0.10** |
| | | 150 | 2.18 | | |
| | | 120 | 2.08 | | |
| | "day end" | 140 | 2.15 | | |
| | | 120 | 2.08 | | |
| | | 130 | **2.11** | | |
| 2 | "day 0" | 150 | 2.18 | 2.11-2.11=0.00 | |
| | | 130 | 2.11 | | |
| | | 130 | **2.11** | | |
| | "day end" | 130 | **2.11** | | |
| | | 140 | 2.15 | | |
| | | 110 | 2.04 | | |
| 3 | "day 0" | 110 | 2.04 | 2.18-2.08=0.10 | |
| | | 130 | 2.11 | | |
| | | 120 | **2.08** | | |
| | "day end" | 150 | **2.18** | | |
| | | 140 | 2.15 | | |
| | | 180 | 2.26 | | |

In this first example, the standard deviation between the 3 results at "day 0" is 0.05 $\log_{10}$ cfu/g for batch 1, 0.04 $\log_{10}$ cfu/g for batch 2, and 0.04 $\log_{10}$ cfu/g for batch 3. Then, all results can be used.
In this example, the highest "δ" value among the 3 batches is 0.10 $\log_{10}$ cfu/g.

A second example of results is shown in Table 5.

**Table 5. Second example of results obtained from a growth potential test.**

CONVERSION in $\log_{10}$

| Batches | Day | Concentration (cfu/g) | Concentration ($\log_{10}$ cfu/g) **In bold: median** | Growth potential "δ" per batch ($\log_{10}$ cfu/g) | Highest"δ" among the 3 batches ($\log_{10}$ cfu/g) |
|---|---|---|---|---|---|
| 1 | "day 0" | 120 | **2.08** | 2.57-2.08 = 0.49 | **0.70** |
| | | 110 | 2.04 | | |
| | | 160 | 2.20 | | |
| | "day end" | 250 | 2.40 | | |
| | | 370 | **2.57** | | |
| | | 380 | 2.58 | | |
| 2 | "day 0" | 160 | 2.20 | 2.74-2.04 = 0.70 | |
| | | 110 | **2.04** | | |
| | | 100 | 2.00 | | |
| | "day end" | 350 | 2.54 | | |
| | | 550 | **2.74** | | |
| | | 620 | 2.79 | | |
| 3 | "day 0" | 120 | 2.08 | 2.48-2.08 = 0.40 | |
| | | 130 | 2.11 | | |
| | | 120 | **2.08** | | |
| | "day end" | 290 | 2.46 | | |
| | | 300 | **2.48** | | |
| | | 330 | 2.52 | | |

The standard deviation between the 3 results at "day 0" is 0.08 $\log_{10}$ cfu/g for batch 1, 0.11 $\log_{10}$ cfu/g for batch 2, and 0.02 $\log_{10}$ cfu/g for batch 3. Then, all results can be used.
In this second example, the highest "δ" value among the 3 batches is retained. In this example, $\delta$ = 0.70 $\log_{10}$ cfu/g.

## j. Exploitation of the results

The FBO is responsible for the use of the results of the challenge test.

### o Ability to support growth of *L. monocytogenes*

The first question to sort out is whether the food is able or not to support the growth of *L. monocytogenes*.

- If δ is lower or equal to the limit of 0.5 $\log_{10}$, then it is assumed that the food is not able to support the growth of *L. monocytogenes*.
- If δ is higher than the limit of 0.5 $\log_{10}$, then it is assumed that the food is able to support the growth of *L. monocytogenes*.

### o Use of the growth potential value

In the cases in which it is assumed that the food is able to support the growth of *L. monocytogenes*, the δ value may be used for predictions of growth (see example), such as:

final concentration = initial concentration + δ

In practice, the final concentration obtained from the calculation may be used to determine for a given product, with a known concentration at "day 0" whether its predicted concentration at "day end" exceeds the limit of 100 cfu/g or not.

### o Examples

QUESTION 1: Does the food support the growth of *L. monocytogenes*, according to the δ value?

EXAMPLE 1

In the first example:

δ = 0.10 $\log_{10}$ cfu/g

δ is below 0.5, then it is assumed that the food does not support growth of *L. monocytogenes*.

EXAMPLE 2

In the second example:

δ = 0.70 $\log_{10}$ cfu/g

δ is higher than 0.5, then it is assumed that the food supports growth of *L. monocytogenes*. This δ-value can be used for further calculations.

QUESTION 2: What is the concentration of *L. monocytogenes* at the end of the test, knowing that the initial concentration is 1 $\log_{10}$ cfu/g?

The predicted concentration of *L. monocytogenes* at the end of the test is:

final concentration = initial concentration + δ

1 + 0.70 = 1.70 $\log_{10}$ cfu/g

(below the legal limit of 2 $\log_{10}$ cfu/g)

## k. Test report

Include in the test report at least the following information:

- ◊ Report number,
- ◊ Purpose of the study
- ◊ Type of challenge test
- ◊ Information concerning full identification of the food:
  - Name of the product,
  - The characteristics of the RTE food (pH, $a_w$, associated microflora, …),
  - The intended shelf-life of the product
  - Identification of the batches and dates of the beginning of the shelf-life.
- ◊ Data relating to the challenge test:
  - Number of batches tested and justification,
  - Number of test units used per batch and per day of analysis,
  - Mass or volume of the test units inoculated,
  - Strains used, strains characteristics (if possible) and justification of the choice,
  - Preparation of the inoculum,
  - Inoculum concentration,
  - Volume of the inoculum introduced per test unit,
  - Contamination method,
  - Date of inoculation,
  - Duration of the test and sampling intervals
  - Storage temperature and justification,
  - Storage duration and justification,
  - Enumeration and detection methods used,
  - Limit of the enumeration,
  - Physico-chemical values at the beginning and at the end of the test,
  - Gas atmosphere,
  - Concentration of associated microflora at the beginning and at the end of the test,
  - Concentration of *L. monocytogenes* at the beginning and at the end of the test,
  - Growth potential per batch,
  - Conclusion.

The results of the challenge test apply only to the product tested. Any change to the product recipe, the process, … would invalidate the results of the shelf-life study and would require it to be conducted again.

### 3.2.2 Challenge test assessing maximum growth rate

#### *3.2.2.1 General aspects*

A microbiological challenge test assessing maximum growth rate is a laboratory-based study which measures the rate of the growth of *L. monocytogenes* in an artificially contaminated food stored at a fixed temperature.

Once the test has been performed, the maximum growth rate ($\mu_{max}$ in natural logarithm) of the *L. monocytogenes* strain at the selected temperature is calculated from the growth curve, one per strain and per batch. In the exponential growth phase, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the $\mu_{max}$. The maximum growth rate is an important parameter of the growth curve which depends on:

- the inoculated strain(s),
- intrinsic properties of the food (e.g. pH, NaCl content, $a_w$, associated microflora, antimicrobial constituents),
- extrinsic properties (e.g. temperature, gas atmosphere, moisture).

Such microbiological challenge tests allow an estimation of the concentration of *L. monocytogenes* at a given day of the shelf-life (especially at the end) if the initial concentration is known.

The advantage of the challenge test assessing maximum growth rate is the flexibility: it is possible to extrapolate a $\mu_{max}$ at a temperature to predict other $\mu_{max}$ values at other temperatures (less than 25°C) in the same food. The disadvantage is that lag time and stationary phase are not taken into account.

#### *3.2.2.2 Protocol of a challenge test to assess maximum growth rate*

The paragraphs "Number of batches", "Measurement of physico-chemical characteristics", "Gas atmosphere" and "Microbiological analyses" are the same as for challenge tests assessing the growth potential (see 3.2.1.2).

##### a. Choice of strains

At least 2 strains are tested separately for each batch. One of them has to be a strain with known growth characteristics (for example EURL *Lm* set of strains). The other strain(s) is (are) freely chosen (for example: from foods, environment, outbreak, collections); knowledge of the growth characteristics is not mandatory for this (these) strain(s).

##### b. Preparation of the inoculum

Conditions for preparation of the inoculum are identical to those described for the challenge test assessing the growth potential except that the strains from the second subculture are not combined in equal quantities but each strain is used individually (Figure 11 in clause 7.3.3).

### c. Preparation and inoculation of the test units

Table 6 shows the minimum number of test units to be prepared per batch.

The whole experiment requires destructive sampling for microbiological procedures.

**Table 6. Minimum number of test units to be prepared per batch**

| | Test units | |
|---|---|---|
| Enumeration of *L. monocytogenes* in inoculated test units | 10 to 15 (with 5 to 7 in exponential phase) | |
| Detection of *L. monocytogenes* in the food in non-inoculated test units | "day 0"<br>3 | "day end"<br>3 |
| Measurement of physical-chemical characteristics in non-inoculated test units | "day 0"<br>1 | "day end"<br>1 |
| Measurement of gas atmosphere (for product under gas atmosphere) in non-inoculated test units | | |
| Enumeration of associated microflora in non-inoculated test units | | |

a) "day 0": the day of inoculation

b) "day end": the end of the shelf-life

An example of the total number of test units is given in clause 7.8.

For measurement of physico-chemical characteristics, gas atmosphere and determination of the concentration of the associated microflora, the same test units may be used.

#### o Preparation and inoculation of test units with *L. monocytogenes*

Conditions for preparation and inoculation of the test units are identical to those described for the challenge test assessing the growth potential except that in the present case the inoculation is performed with one strain (not a mixture) for each growth curve.

### d. Storage conditions for the inoculated food

The challenge test is conducted at a fixed temperature.

### e. Calculation of the maximum growth rate

Different tools may be used for estimating the maximum growth rate[5]. One of them, freely available DMFit, is described below.

[5] The result « $\mu_{max}$ » is expressed in natural logarithm and the result « rate » is expressed in decimal logarithm. $\mu_{max}$ (in natural logarithm) = ln 10 x rate (in decimal logarithm)

The results of the enumeration are calculated according to the Standard EN ISO 7218 and expressed in decimal logarithm cfu/g ($\log_{10}$ cfu/g).

For each curve (i.e. all the experimental points from one batch) the growth rate can be easily estimated by non-linear regression[6]. A software as DMFit can be used for that purpose. The DMFit software (Figure 5) chooses, between different models, the one with the best fitting.

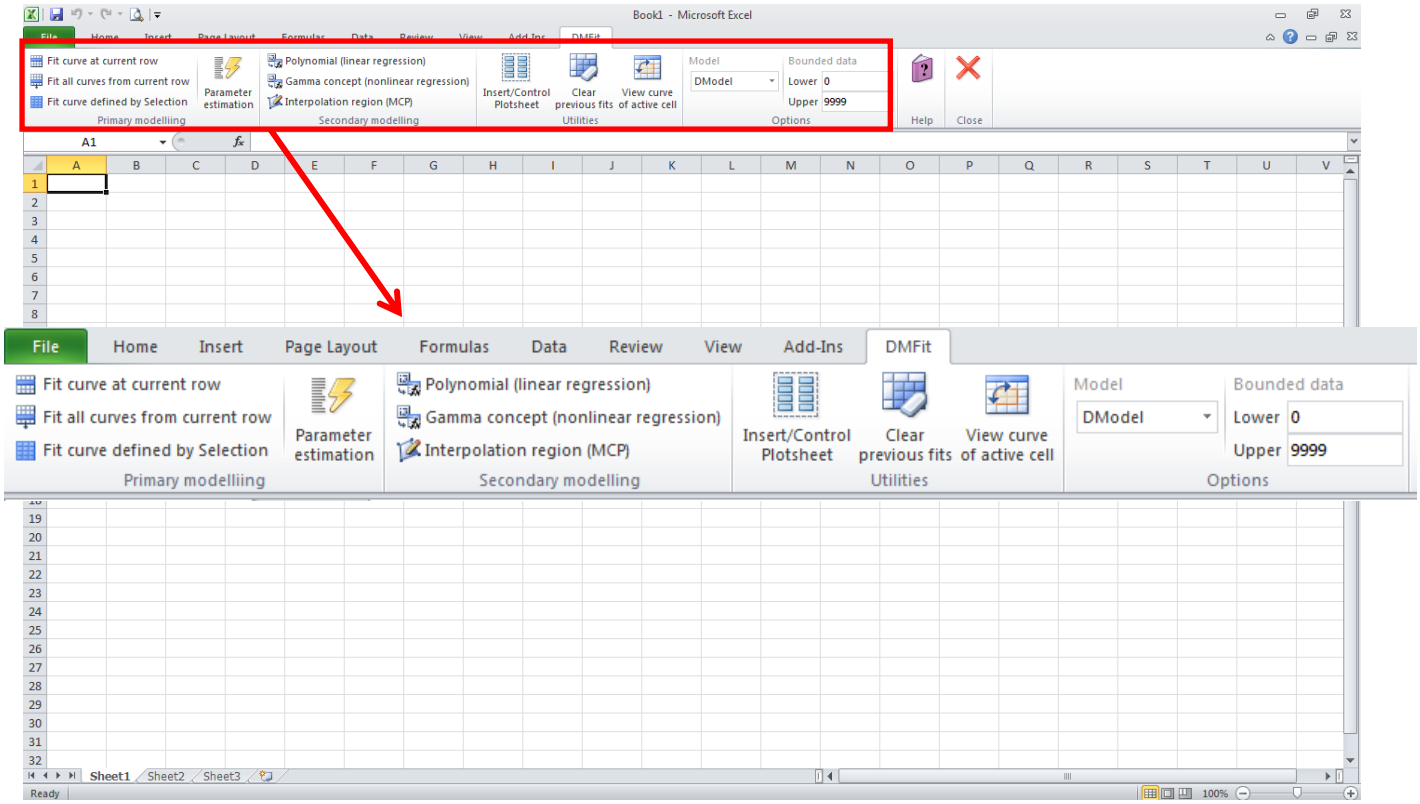



**Figure 5. DMFit software to fit a growth curve**

The DMFit software provides a chart (Figure 6) with the experimental points and the curve fitted by regression, using the most adapted model. It also extracts the growth parameters of the curve, in particular the growth rate named "rate" (expressed in decimal logarithm), in the sheet Curve1. The parameter "lag" is also calculated but with this protocol, lag phase is only dependent on temperature, so this data is taken with caution.

[6] A linear regression can also be used to estimate the growth rate: at least 5 days of analysis in exponential phase are planned, with 3 test units per day of analysis

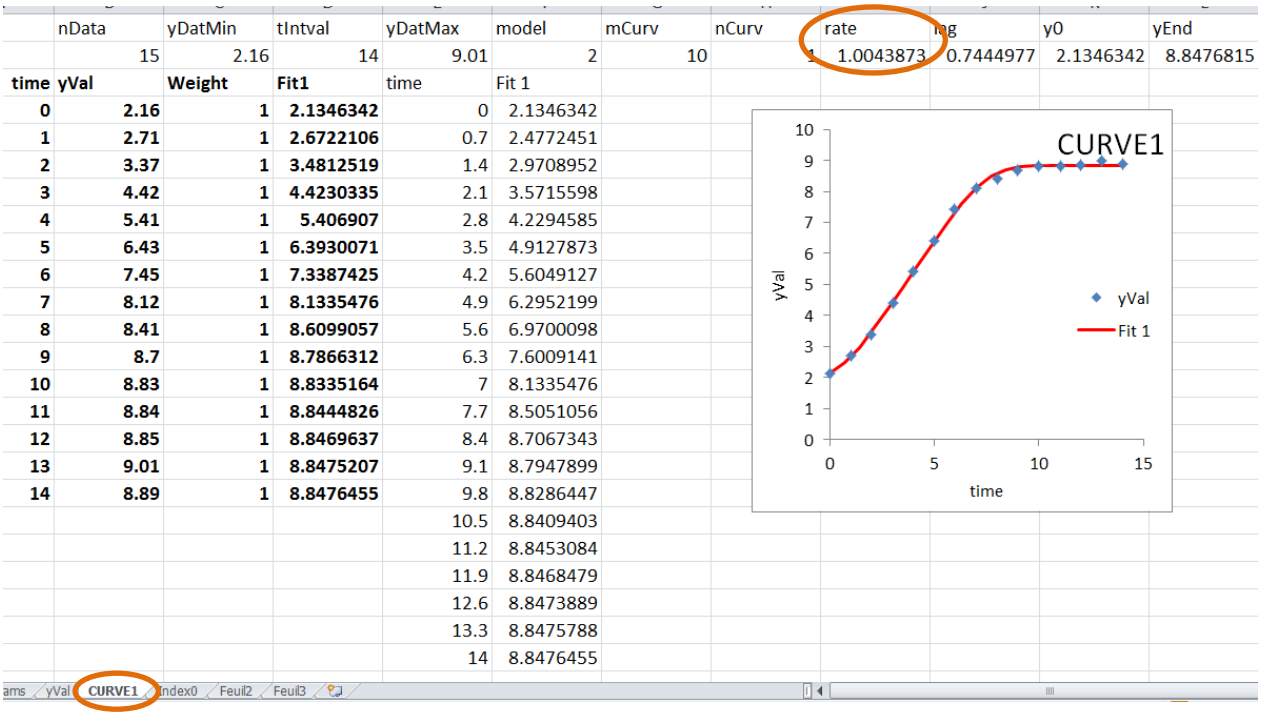



**Figure 6. An example of fitting**

Estimated standard deviations are calculated in particular for rate (Figure 7), in the sheet Index0. Estimated standard deviation for rate (σ) is used to calculate the highest limit of the confidence interval (+2σ at α=95%) for the rate.

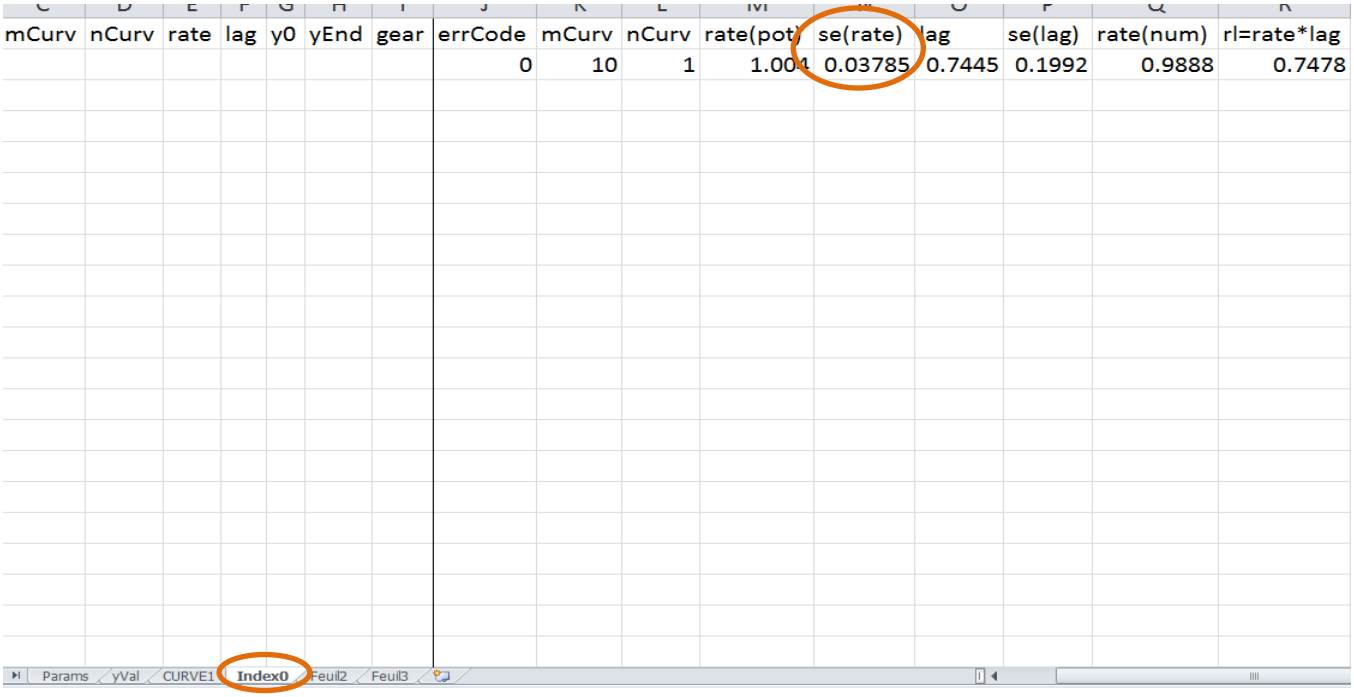



**Figure 7. An example of estimated standard deviation for a fitting by DMFit**

For more information related to the use of DMFit, a manual is available when downloading the software.

For each growth curve, a rate is calculated. If using 1 batch and 2 strains, then interpret 2 growth curves. If using 3 batches and 2 strains, then interpret 6 growth curves.
Then retain the growth curve with the highest growth rate value for the exploitation of results.

## f. Exploitation of the results

The FBO is responsible for the implementation of the results of the challenge test.

### o Growth rate

Knowing the growth rate value at a temperature ($T_{ref}$), it is possible to calculate another growth rate at another temperature (T). Thus, from a growth curve at $T_{ref}$, the estimated rate using DMFit is denoted $rate_{ref}$. Then, the calculation of rate in the same food (with the same physico-chemical characteristics) at another temperature T will be obtained using the square-root secondary model. If T and $T_{ref}$ are both inferior to 25°C, the following simplified formula is suggested:

$$rate = rate_{ref} \cdot \frac{(T - T_{min})^2}{(T_{ref} - T_{min})^2}$$

(with $T_{min}$ = minimal growth temperature for *L. monocytogenes* ≈ -1.5°C)

$rate_{ref}$ and rate may be expressed in $\log_{10}$ cfu/g per time unit.
Other secondary models may be used.

### o Growth determination

Assuming a very simple primary model (without lag phase nor stationary phase, which may lead to fail-safe result):
Growth ($\log_{10}$) obtained at $T_{ref}$ during a storage time $d_1$ (in days) = $rate_{ref}$ ($\log_{10}$ cfu/g per day) x $d_1$
Growth ($\log_{10}$) obtained at T during a storage time $d_2$ (in days) = rate ($\log_{10}$ cfu/g per day) x $d_2$
Other primary models may be used.

The prediction can then be applied to any time-temperature profile, and in particular to the conditions at which the product is most likely to be subjected in normal use, until its final consumption.

- Example

  - **Data**:

- Shelf-life: 9 days, "day 0" is the day of production

- Storage conditions: 4°C for 3 days ($d_1$) and 8°C for 6 days ($d_2$)

The challenge test was performed at $T_{ref}$ = 8°C.

- $rate_{ref}$ = 1.00 $\log_{10}$ cfu/g (Figure 6)

The maximal $rate_{ref}$ could be obtained: $rate_{ref_{max}}$ = 1.00 + (2 x 0.04) = 1.08 $\log_{10}$ cfu/g per day (Figure 7).

The secondary model enables to predict rate at T = 4°C

$$rate_{max} = rate_{ref_{max}} \cdot \frac{(T - T_{min})^2}{(T_{ref} - T_{min})^2}$$

The point-estimate is:

$$rate_{max} = [1.08 \frac{(4 - (-1.5))^2}{(8 - (-1.5))^2}]$$
$$= 0.36 \log_{10} \text{cfu/g per day}$$

Then, the maximum growth rate predicted at 4°C is 0.36 $\log_{10}$ cfu/g per day.

- **Question 1:** what is the growth of *L. monocytogenes* predicted during the shelf-life?

Growth during the shelf-life =

[($rate_1$ in $\log_{10}$ cfu/g per day) x $d_1$] + [($rate_2$ in $\log_{10}$ cfu/g per day) x $d_2$] where:

$$Growth_{max} = (0.36 \times 3) + (1.08 \times 6)$$

$$= 7.56 \log_{10} \text{cfu/g}$$

This calculation does not include the lag phase and the stationary phase (i.e. assumes the whole simulated behaviour is exponential growth), and consequently, the results may be (very) fail-safe.

- **Question 2:** what is the concentration of *L. monocytogenes* at the end of the shelf-life if the level for *L. monocytogenes* at day 7 is equal to 1.65 $\log_{10}$ cfu/g?

The maximum level of *L. monocytogenes* at "day end" will be 1.65 + 1.08 x 2 = 3.81 $\log_{10}$ cfu/g).

The limit of 100 cfu/g is exceeded for this product.

## g. Test report

Include in the test report at least the following information:

- ◊ Report number,
- ◊ Purpose of the study
- ◊ Type of challenge test
- ◊ Information concerning full identification of the food:
  - Name of the product,
  - The characteristics of the RTE food (pH, $a_w$, associated microflora, …),
  - The intended shelf-life of the product
  - Identification of the batches and dates of the beginning of the shelf-life.
- ◊ Data relating to the challenge test:
  - Number of batches tested and justification,
  - Number of test units used per batch and per day of analysis,
  - Mass or volume of the test units inoculated,
  - Strains used, strains characteristics (if possible) and justification of the choice,
  - Preparation of the inoculum,
  - Inoculum concentration,
  - Volume of the inoculum introduced per test unit,
  - Contamination method,
  - Date of inoculation,
  - Duration of study and sampling intervals
  - Storage temperature
  - Enumeration and detection methods used,
  - Limit of the enumeration,
  - Physico-chemical values at the beginning and at the end of the test,
  - Gas atmosphere,
  - Concentration of associated microflora at the beginning and at the end of the test,
  - Concentration of *L. monocytogenes* along the test,
  - Fitting curve with experimental points
  - Maximum growth rate per batch,
  - Conclusion.

The results of the challenge test apply only to the product tested. Any change to the product recipe, the process, … would invalidate the results of the shelf-life study and would require this study to be conducted again.

# 4 Durability study

To conduct a durability study, the following points have to be considered: the sampling procedure, the storage conditions and the enumeration method for *L. monocytogenes*.

## 4.1 Food sampling procedure

### 4.1.1 Introduction

The sampling procedure has to take into account the heterogeneity of the production.

For this purpose, ISO Standards and Codex Alimentarius General Guidelines on Sampling (CAC/GL 50-2004) shall be used as reference methods as indicated in Chapter 3, clause 3.1 of Annex I to Regulation (EC) No. 2073/2005.

When no information on the structure of the batch is available, the most objective way to draw test units is to give all the test units of the production the same chance to be drawn. The simple random sampling is recommended to estimate the proportion of test units above the limit of 100 cfu/g.

### 4.1.2 Simple random sampling

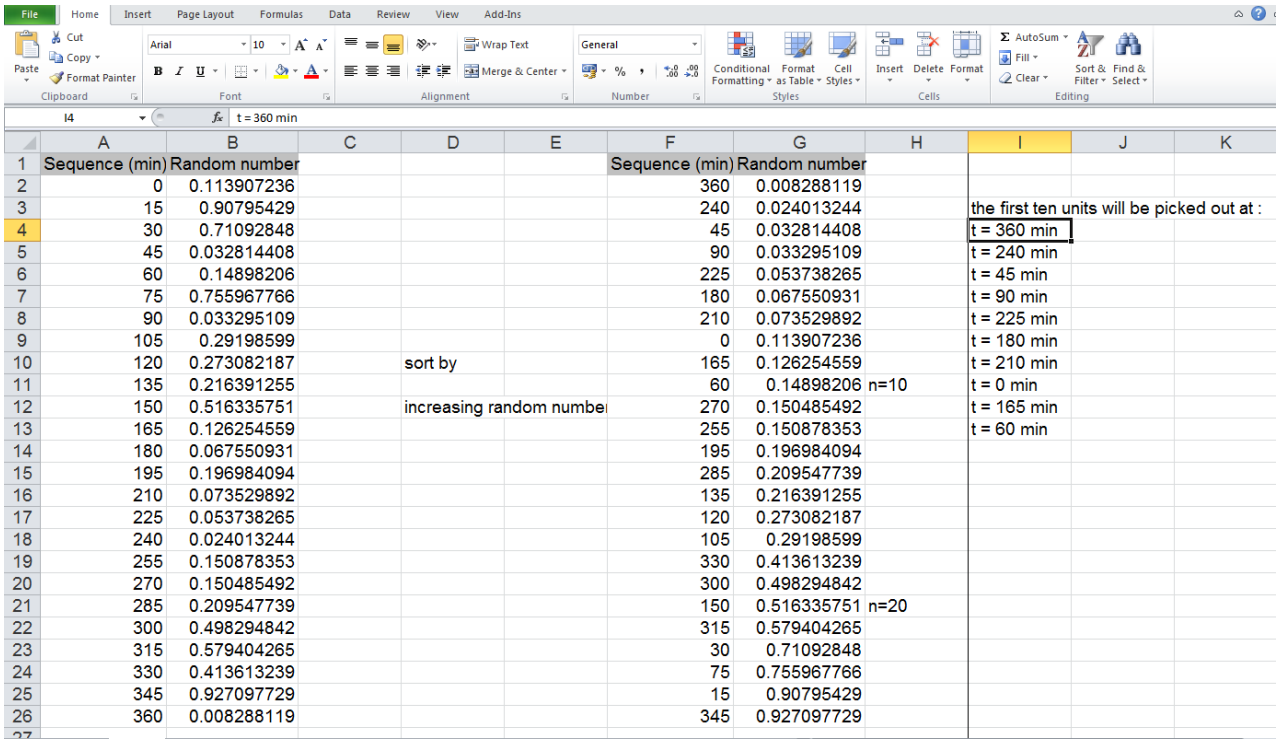
#### *4.1.2.1 Description*

This sampling method is based on the equiprobability principle. This principle guarantees each unit of the batch to have an equal chance of being selected. To satisfy this principle, the size of the batch (N) has to be large enough in comparison to the number (*n*) of test units: n / N < 10%.

One way of achieving simple random sampling is to number each unit of the batch or in a more practical way the "production time" and then to use random numbers to select the required number of test units. For example, random numbers can be obtained with an Excel sheet with the formula =RAND( ) (see Figure 8), or from random number tables.

#### *4.1.2.2 Example of a method used to select randomly 10 test units from a batch*

Given that the time for producing 1 batch is 6 h, these 6 h could be divided into periods of 15 min. Enter this sequence of 15 in an Excel sheet and select the random function to give a random number to each sequence. These random numbers are then classified by increasing numbers and the first ten ones are selected. Then, a person in charge of sampling will draw at the end of the production line the ten units at the selected times.



| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Sequence (min) | Random number | | | | Sequence (min) | Random number | | | | |
| 2 | 0 | 0.113907236 | | | | 360 | 0.008288119 | | | | |
| 3 | 15 | 0.90795429 | | | | 240 | 0.024013244 | | the first ten units will be picked out at : | | |
| 4 | 30 | 0.71092848 | | | | 45 | 0.032814408 | | t = 360 min | | |
| 5 | 45 | 0.032814408 | | | | 90 | 0.033295109 | | t = 240 min | | |
| 6 | 60 | 0.14898206 | | | | 225 | 0.053738265 | | t = 45 min | | |
| 7 | 75 | 0.755967766 | | | | 180 | 0.067550931 | | t = 90 min | | |
| 8 | 90 | 0.033295109 | | | | 210 | 0.073529892 | | t = 225 min | | |
| 9 | 105 | 0.29198599 | | | | 0 | 0.113907236 | | t = 180 min | | |
| 10 | 120 | 0.273082187 | | sort by | | 165 | 0.126254559 | | t = 210 min | | |
| 11 | 135 | 0.216391255 | | | | 60 | 0.14898206 | n=10 | t = 0 min | | |
| 12 | 150 | 0.516335751 | | increasing random numbe | | 270 | 0.150485492 | | t = 165 min | | |
| 13 | 165 | 0.126254559 | | | | 255 | 0.150878353 | | t = 60 min | | |
| 14 | 180 | 0.067550931 | | | | 195 | 0.196984094 | | | | |
| 15 | 195 | 0.196984094 | | | | 285 | 0.209547739 | | | | |
| 16 | 210 | 0.073529892 | | | | 135 | 0.216391255 | | | | |
| 17 | 225 | 0.053738265 | | | | 120 | 0.273082187 | | | | |
| 18 | 240 | 0.024013244 | | | | 105 | 0.29198599 | | | | |
| 19 | 255 | 0.150878353 | | | | 330 | 0.413613239 | | | | |
| 20 | 270 | 0.150485492 | | | | 300 | 0.498294842 | | | | |
| 21 | 285 | 0.209547739 | | | | 150 | 0.516335751 | n=20 | | | |
| 22 | 300 | 0.498294842 | | | | 315 | 0.579404265 | | | | |
| 23 | 315 | 0.579404265 | | | | 30 | 0.71092848 | | | | |
| 24 | 330 | 0.413613239 | | | | 75 | 0.755967766 | | | | |
| 25 | 345 | 0.927097729 | | | | 15 | 0.90795429 | | | | |
| 26 | 360 | 0.008288119 | | | | 345 | 0.927097729 | | | | |

Figure 8: Example of a random sampling scheme with an Excel sheet

This sampling method should be repeated for different batches (same product, produced under similar conditions) to obtain representative data.

## 4.2 Storage conditions

See the part “Storage conditions” in the clause 3.2.1.2 “Challenge test assessing growth potential”.

## 4.3 Microbiological analyses

At the end of the storage period, all the units are analysed with the enumeration method in order to assess whether the level of 100 *Listeria monocytogenes*/g is exceeded or not.

According to Annex I of Regulation No. 2073/2005, the reference enumeration method for *L. monocytogenes* is the Standard method EN ISO 11290-2, amended. According to Article 5 of the same regulation, the use of alternative analytical methods is acceptable when the methods are validated against the reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO Standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the Competent Authority.

The enumeration limit should be 10 cfu/g, in order to be able to precisely quantify the contamination level of *L. monocytogenes* at the end of the storage period (see 3.2.1.2.h).

## 4.4 Calculation

In the case of batch testing, the criterion defined by Regulation No. 2073/2005 is "n=5, c=0, m=M=100 cfu/g at the time of consumption". When the limit defined by the criterion is exceeded, the product is considered not to be safe and cannot be put on the market. Revision and improvement of the production process is thus required.

However, such batch conformity controls are not in the scope of the present document.

The interpretation of durability studies, which consists in verifying that the limit of 100 cfu/g is not exceeded at the time of consumption, is a different case. As described below, this interpretation can be facilitated by the assessment of the proportion of units exceeding 100 cfu/g (with its associated confidence interval) at the end of shelf-life, after a storage period reflecting the foreseeable conditions of distribution and storage.

From the number ($n$) of test units taken randomly from a batch (of size $N$), the estimated proportion ($p$) of units exceeding 100 cfu/g at the end of shelf-life is derived simply as the observed proportion $p = r / n$ (where $r$ is the number of test units above 100 cfu/g).

To calculate the confidence interval associated to the estimated proportion ($p$), a calculator can be used. Numerous calculators are freely available on the internet, for example: http://www.causascientia.org/math_stat/ProportionCI.html. This calculator proposes two methods of calculation, the central confidence interval or the shortest confidence interval. Confidence intervals given by each method may be slightly different but are in the same order of magnitude.

On the tested units, after the storage period, Table 7 gives estimated proportions ($p$) with their associated confidence intervals for three values of ($r$) (number of units > 100 *L. monocytogenes* / g).

Table 7 points out the real importance of drawing from one batch a sufficient number of units, and/or to gather results previously obtained, to get a correct estimation of the proportion of test units greater than 100 cfu/g, with a reduced confidence interval.

**Table 7 : Example of estimated proportion of test units > 100 *L. monocytogenes*/g after storage period**

| *n*<br>number of analysed test units | *r*<br>number of test units > 100 cfu/g | *p*<br>estimated proportion | CI<br>Confidence Interval at 95% |
|---|---|---|---|
| 20 | 0 | 0% | [0% – 16%] |
| 100 | | 0% | [0% – 4%] |
| 20 | 1 | 5% | [1% – 24%] |
| 100 | | 1% | [0.2% – 5%] |
| 20 | 2 | 10% | [3% – 30%] |
| 100 | | 2% | [0.6% – 7%] |

The more units are analysed, the narrower the confidence interval is. For example, it can be concluded from Table 7 that the upper limit of the confidence interval for "2 test units exceeding 100 cfu/g out of 100 test units" is lower than that obtained for "0 test units exceeding 100 cfu/g out of 20 test units".
To get a large number of analysed units, it is possible to gather results of repeated tests, performed on one RTE food obtained from the same process. For example, it can be seen in Table 8 that the upper limit of the confidence interval for "0 test units exceeding 100 cfu/g out of 5 test units" is higher than that obtained for "0 test units exceeding 100 cfu/g out of 30 test units".

**Table 8: Example of confidence interval at 95% with regard to the number of analysed test units**

| *n*<br>number of analysed test units | *r*<br>number of test units > 100 cfu/g | *P* [CI]<br>estimated proportion [Confidence Interval at 95%] |
|---|---|---|
| 5 | 0 | 0% [0% – 46%] |
| 10 | 0 | 0% [0% – 28%] |
| 15 | 0 | 0% [0% – 21%] |
| 20 | 0 | 0% [0% – 16%] |
| 25 | 0 | 0% [0% – 13%] |
| 30 | 0 | 0% [0% – 11%] |
| … | 0 | … |
| 95 | 0 | 0% [0% – 3.8%] |
| 100 | 0 | 0% [0% – 2.9%] |
| . . . | 0 | . . . |

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# 6 Glossary

Abuse temperature: temperature higher than the prescribed temperature of processing and retail storage according to national legislated temperature rules or European food regulations, including reasonably foreseeable domestic storage conditions. The abuse temperature covers the whole cold chain, taking into account in particular the temperature deviation of retail refrigerators as well as domestic storage.

Batch: group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period. (Regulation (EC) No. 2073/2005).

Challenge test: study of the evolution of microorganisms populations artificially inoculated in a food.

Cold chain: the continuous system that provides chilled storage of perishable foods, from production to consumption.

Durability study: study of the evolution of microorganisms populations naturally present in a food.

Exponential phase: growth phase when the bacterial population grows at a constant maximum specific rate.

Growth potential: difference between the decimal logarithm of the final concentration of a microbial population and the decimal logarithm of the initial concentration of this microbial population.

Hygrometry: measurement of the moisture in air and gases.

Lag phase: phase when the bacterial population is in an adaptation period to the growth environment, without visible growth, before bacteria arrive at the exponential phase.

Percentile: the $x^{th}$ percentile of a set of values divides these values so that x% of the values lie below and (100-x)% of the values lie above. Examples: Ninety percent of the values lie at or below the ninetieth percentile, ten percent above it. The median of the values corresponds to the $50^{th}$ percentile, that is fifty percent of the values below the median and fifty percent above the median.

pH: a measure of the acidity or alkalinity of a food. The pH 7 is defined as neutral. Values of a pH less than seven are considered acidic and those with greater than seven are considered basic (alkaline).

$rate_{max}$: slope of the curve showing the evolution of the decimal logarithm of the population according to the time during the exponential phase.

Ready-to-eat (RTE) food: food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level microorganisms of concern.

Sampling: procedure used to draw or selection of one or more unit(s).

Shelf-life: either period corresponding to the period preceding the 'use by' or the minimum durability date, as defined respectively in articles 9 and 10 of Directive 2000/13/EC concerning, among others, the labelling of foodstuffs.

Stationary phase: phase when the bacterial population reaches the maximum carrying capacity of the environment, after the exponential phase.

Surrogate organism: alternative organism

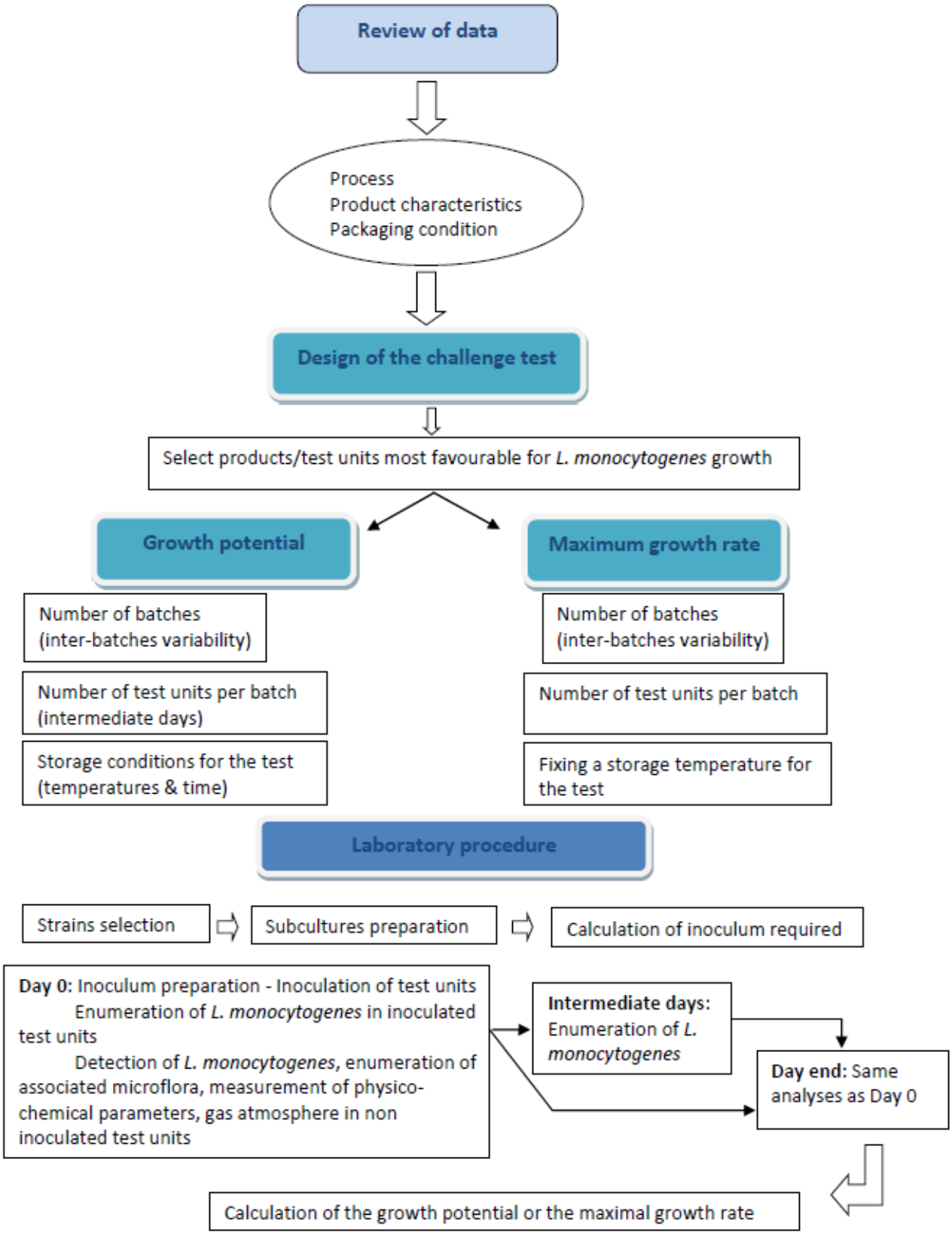
Test unit: aliquot of a commercial unit, designed to be analysed.

$\mu_{max}$: slope of the curve showing the evolution of the natural logarithm of the population according to the time during the exponential phase.

Water activity ($a_w$): the term refers to the unbound and available water in a food and is not the same as the water content of the food. Water in food which is not bound to other molecules can support the growth of microbes. The water activity scale extends from 0 to 1.0 (pure water) but most foods have a water activity level in the range of 0.2 for very dry foods to 0.99 for moist fresh foods.

# 7 Annexes

## 7.1 Flow diagram describing schematically the steps from review of data to test in the laboratory



## 7.2 EURL Lm set of *L. monocytogenes* strains with their growth characteristics

The strains set of EURL Lm was classified according to their growth rates related to origins, conditions of temperature, pH and $a_w$, and genoserotypes. More details are described in the report dedicated to strains set for challenge tests (available on http://www.ansespro.fr/eurl-listeria/index.htm).

**Table 9. Choice of strains according to growth abilities related to origins, conditions and genoserotypes**

| Origin | Meat products | | |
|---|---|---|---|
| Genoserotype | Low $a_w$ ($a_w$ = 0.95) | Low pH (pH = 5) | Low temperature (T = 8°C) |
| II | 12MOB045LM<br>12MOB046LM | 12MOB045LM<br>12MOB046LM | 12MOB045LM<br>12MOB046LM |
| IV | 12MOB085LM<br>12MOB089LM | 12MOB112LM<br>12MOB089LM | 12MOB085LM<br>12MOB089LM |
| Origin | Fish products | | |
| Genoserotype | Low $a_w$($a_w$ = 0.95) | Low pH (pH = 5) | Low temperature (T = 8°C) |
| II | 12MOB101LM<br>12MOB100LM | 12MOB101LM<br>12MOB100LM | 12MOB099LM<br>12MOB101LM |
| IV | 12MOB103LM<br>12MOB102LM | 12MOB103LM<br>12MOB102LM | 12MOB102LM<br>12MOB107LM |
| Origin | Dairy products | | |
| Genoserotype | Low $a_w$ ($a_w$ = 0.95) | Low pH (pH = 5) | Low temperature (T = 8°C) |
| II | 12MOB098LM<br>12MOB118LM | 12MOB118LM<br>12MOB098LM | 12MOB098LM<br>12MOB079LM |
| IV | 12MOB053LM<br>12MOB106LM | 12MOB053LM<br>12MOB096LM | 12MOB096LM<br>12MOB105LM |
| Origin | Other products | | |
| Genoserotype | Low $a_w$ ($a_w$ = 0.95) | Low pH (pH = 5) | Low temperature (T = 8°C) |
| II | 12MOB048LM<br>12MOB047LM | 12MOB051LM<br>12MOB047LM | 12MOB049LM<br>12MOB047LM/<br>12MOB051LM |
| IV | 12MOB050LM<br>12MOB052LM | 12MOB050LM<br>12MOB052LM | 12MOB052LM<br>12MOB050LM |

How to use this table?

Example 1: If the product to be tested comes from dairy products, is rather acid (pH ≤ 5), then the chosen strain could be 12MOB118LM or 12MOB098LM or 12MOB053LM or 12MOB096LM if the genoserotype is unknown.

Example 2: If the product to be tested comes from meat products, is neither acid (pH > 5), neither with a low $a_w$ ($a_w$ > 0.95), then the chosen strain could be 12MOB045LM or 12MOB046LM or 12MOB085LM or 12MOB089LM if the genoserotype is unknown.

## 7.3 Example of preparation of the inoculum for the challenge test

### 7.3.1 Preparation of subcultures for strain 1

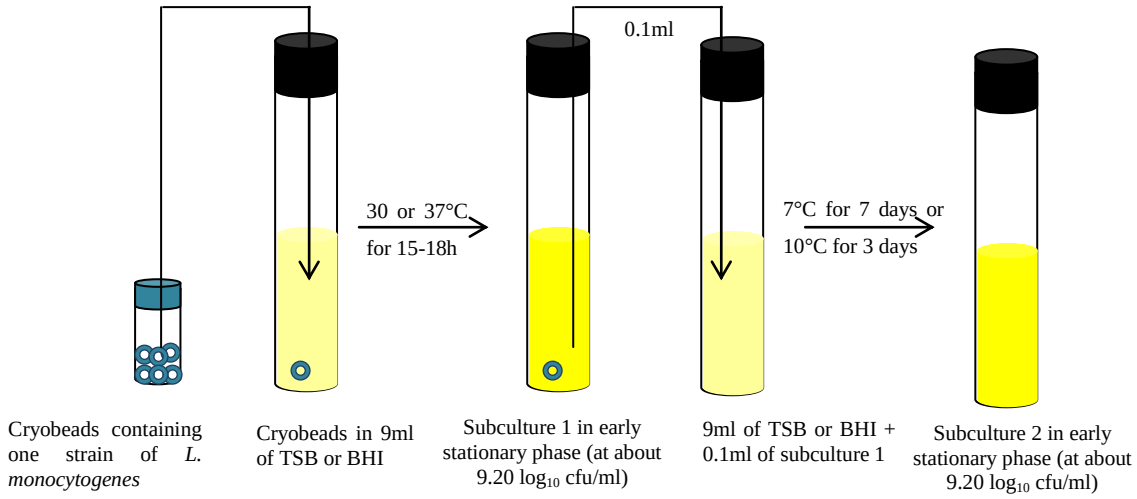



Figure 9: Preparation of the 2 subcultures for each strain

Process is repeated for strain 2 and other strains if used. Values given are for EURL Lm strains.

### 7.3.2 Preparation of the inoculum for challenge test assessing growth potential

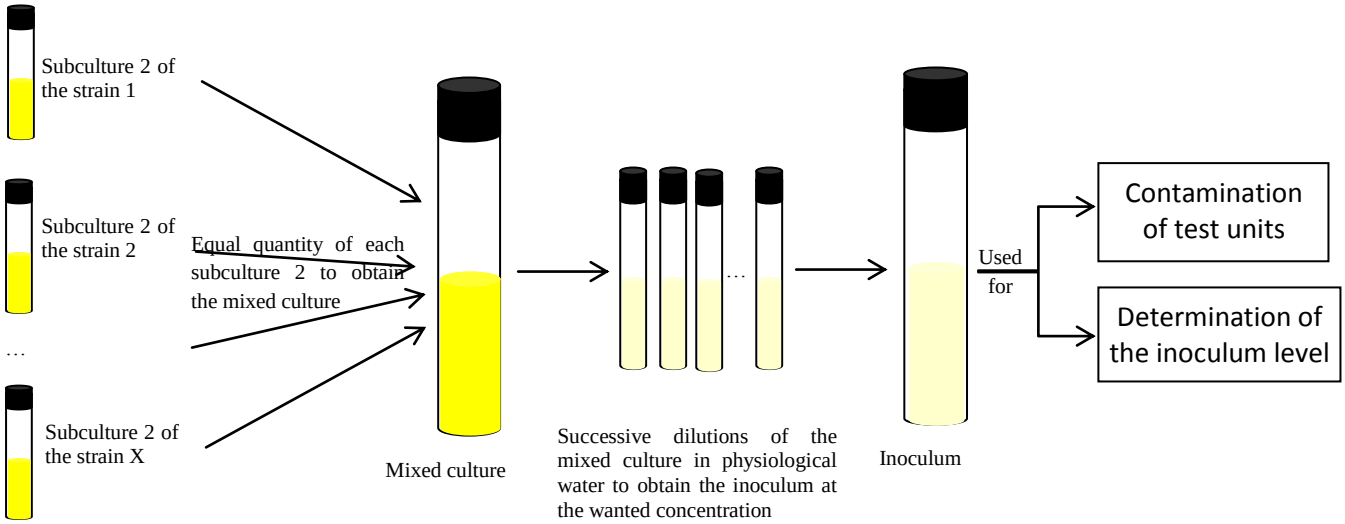



Figure 10: Preparation of the inoculum from the subcultures 2

### 7.3.3 Preparation of the inoculum for challenge test assessing maximum growth rate

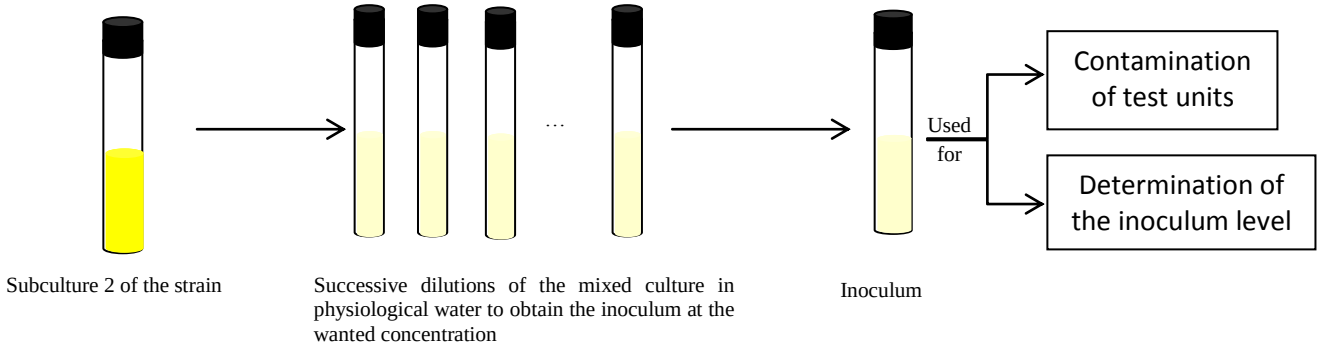



Figure 11: Preparation of the inoculum from the subculture 2 of one strain

### 7.3.4 Method to obtain the targeted concentration of the inoculum with a numerical example:

The mixed culture for challenge test assessing growth potential has an estimated concentration of 9.2 $\log_{10}$ cfu/ml, that is $1.58.10^9$ cfu/ml.

The targeted concentration in the whole matrix is 100 cfu/g.

The mass of the whole matrix is 650g. The volume of the inoculum should not exceed 1% of the mass of the whole matrix; the maximum volume of the inoculum is 6.5ml.

It is necessary to dilute four times by decimal dilutions the mixed culture to come close to the required concentration of the inoculum in the whole matrix: $C_{\text{mixed culture diluted}} = 1.58.10^5$ cfu/ml.

It is necessary to prepare a larger quantity of the inoculum to contaminate the matrix (> 6.5 ml). For example 10 ml, so the concentration of the inoculum is $1.58.10^4$ cfu/ml.

The next step is to determine the required volume of the inoculum in order to contaminate the 650 g of the matrix. It is known that:

$$C_{\text{inoculum}} \times V_{\text{inoculum}} = C_{\text{whole matrix}} \times M_{\text{matrix}}$$

$$V_{\text{inoculum}} = (C_{\text{whole matrix}} \times M_{\text{matrix}}) / C_{\text{inoculum}}$$

$$V_{\text{inoculum}} = (100 \text{ cfu/g} \times 650 \text{ g}) / 1.58.10^4$$

$$V_{\text{inoculum}} = 4.11 \text{ ml}$$

In summary:

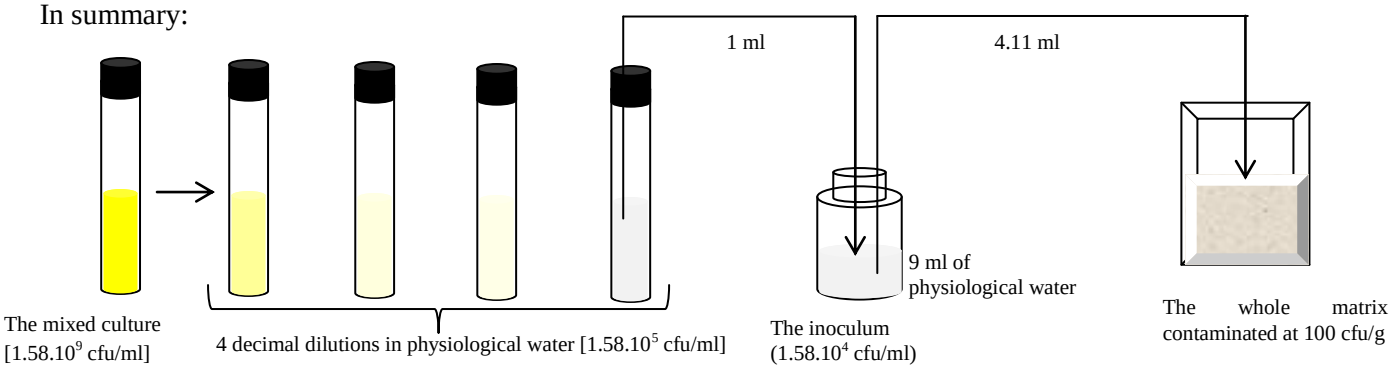



Figure 12: From the mixed culture to the inoculation of the whole matrix

The method to obtain the targeted concentration of the inoculum is the same for challenge test assessing the maximum growth rate except that the targeted inoculum has to be prepared for each of the strains (strains 1, 2, …, X if used) from the second subculture.

## 7.4 Examples of total number of test units required per batch in the frame of a challenge test to assess a growth potential

**Table 10. Total number of test units to be prepared per batch depending on the number of days of analysis in the frame of a challenge tests to assess a growth potential**

| | | "day 0" | Optional intermediate days | | | "day end" |
|---|---|---|---|---|---|---|
| Enumeration of *L. monocytogenes* in inoculated test units | | 3 | 3 | 3 | 3 | 3 |
| Detection of *L. monocytogenes* in non-inoculated test units | | 3 | 0 | 0 | 0 | 3 |
| Measurement of physico-chemical characteristics<br>Enumeration of the associated microflora | | 1 | 0 | 0 | 0 | 1 |
| Total number required per batch | at"day 0" and "day end" | 14 | | | | |
| | with "day 0", 1 intermediate day and "day end" | 17 | | | | |
| | with "day 0", 2 intermediate days and "day end" | 20 | | | | |
| | with "day 0", 3 intermediate days and "day end" | 23 | | | | |

## 7.5 Some examples of contamination techniques

Test units can be contaminated in depth or on surface.

This paragraph gives some examples of a couple of matrix and inoculation technique.

- In depth: a semi-liquid product in small quantity (20 g) in a sterile bag

  for example 20 g of custard contaminated

  by a pipetted volume

- In depth: a semi-liquid product in large quantity (≈500 g) with a blender bowl and then divided in x samples of x g

  for example custard in large quantity contaminated

  by a pipetted volume

- At the surface: a sliced product

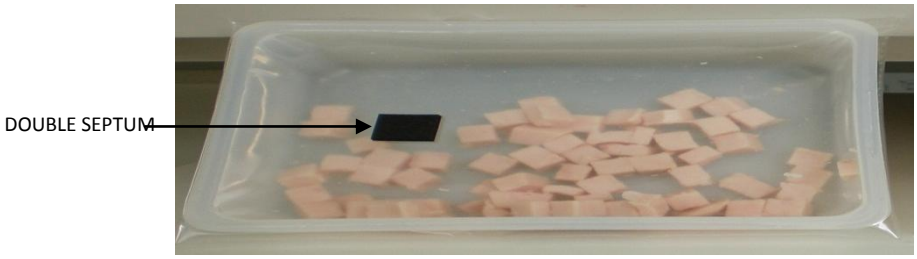
  for example a slice of smoked salmon contaminated with 5 spots of 20 µl on half of the disk's surface and then the disk is folded over. A spreader is used to improve the distribution of the inoculum.

- At the surface: a solid product of small pieces

  for example shredded ham

  contaminated at the surface of pieces with a graduated syringe through a septum. This septum is immediately recovered by a second septum in order not to break packaging atmosphere and maintaining the exact gas conditions.

  Note: It is possible to divide the inoculum into 2 parts and dispatched through 2 septums. The inoculum could be divided into more parts and dispatch through more septums. After inoculation, test units are shaken a lot in order to distribute homogeneously the inoculum.




## 7.6 Example of the impact of storage temperature on the shelf-life

Temperature during the shelf-life is a critical part of the challenge test assessing the growth potential. This is illustrated below on a meat product stored at different temperatures:

- Scenario #1: a constant temperature at 4°C;
- Scenario #2: includes 3 steps (one third of the shelf-life for each step), (i) 4°C to mimic transportation from plant to retail, (ii) 8°C to mimic storage at retail and (iii) 8°C to mimic storage at consumer;

- Scenario #3: includes 3 steps (one third of the shelf-life for each step), (i) 8°C to mimic transportation from plant to retail, (ii) 12°C to mimic storage at retail and (iii) 12°C to mimic storage at consumer.

Shelf-life of the product: 31 days.

Physico-chemical characteristics of the product:

- pH = 6.1 and
- $a_w$ = 0.978.

Packaging of the product: 50% $CO_2$ / 50% $N_2$.

Contaminated portion: 100g.

Mean initial contamination level of *Listeria monocytogenes* in this product: -2 $\log_{10}$ cfu/g.

The shelf-life of the product is estimated for each scenario (Figure 13).

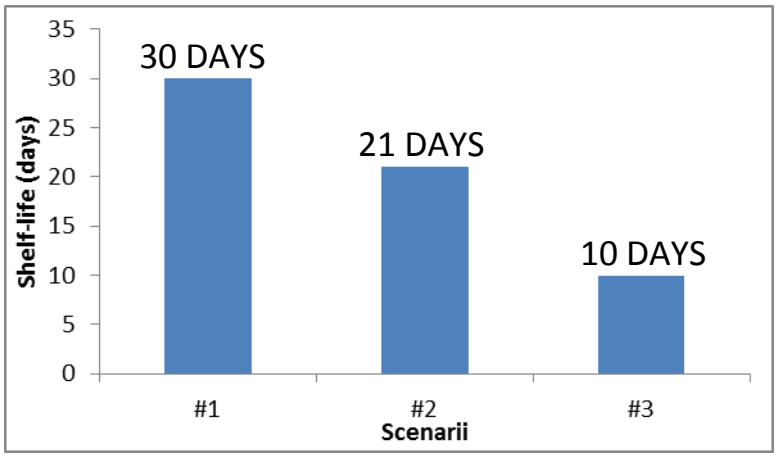



Figure 13: Shelf-life of a meat product related to the different scenarii

The shelf-life of the product is 30 days in the scenario #1. The shelf-life obtained for scenarii #2 and #3 are respectively1.4 and 3 times shorter.

## 7.7 Example of preparation of the initial suspension

The total quantity of the test unit has to be analysed after artificial inoculation.

In case of a large quantity of the test unit, the initial suspension can be prepared by:

- portioning the test units and analysing all the portions, or;
- analysing the entire portion and preparing the initial suspension in 2 steps: performing 2 successive dilutions, for example the 1st dilution in half and then the 2nd dilution at 1/5. For an example: the first dilution is made by taking 50 g of the matrix with 50 ml of the diluent. They are mixed and then for the second dilution, 20 g of the first dilution in half are diluted with 80 ml of the diluent.

## 7.8 Examples of the total number of test units to be prepared in the frame of a challenge test to assess a maximum growth rate, per strain, per batch

**Table 11. Total number of test units to be prepared in the frame of a challenge tests to assess a maximum growth rate, per strain, per batch**

| | 1 batch | 2 batches | 3 batches | … | X batches |
|---|---|---|---|---|---|
| 1 strain | 18 to 21 test units | 36 to 42 test units | 54 to 63 test units | … | 18X to 21X test units |
| 2 strains | 36 to 42 test units | 72 to 84 test units | 108 to 126 test units | … | 36X to 42X test units |
| … | … | … | … | … | … |
| Y strains | 18Y to 21Y test units | 36Y to 42Y test units | 54Y to 63Y test units | … | 18XY to 21XY test units |